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Rapid In Situ Assay for Indoleacetic Acid Production by Bacteria Immobilized on a Nitrocellulose Membrane

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We have developed a new assay that differentiates between indoleacetic acid (IAA)-producing and -nonproducing bacteria on a colony plate lift. Medium supplemented with 5 mM L-tryptophan is inoculated with isolates of interest, overlaid with a nitrocellulose membrane, and then incubated until bacterial colonies reach 1 to 2 mm in diameter. The membrane is removed to a filter paper saturated with Salkowski reagent and incubated until distinct red haloes form around the colonies. The colorimetric reaction to IAA is limited to a region immediately surrounding each colony, is specific to isolates producing IAA, occurs within 1 h after the membrane is placed in the reagent, and is sensitive to as little as 50 pmol of IAA in a 2-mm^2 spot. We have used this assay for quantifying epiphytic and endophytic populations of IAA-producing isolates of *Pseudomonas syringae* subsp. *savastanoi* and for detecting IAA-producing colonies of other pseudomonads and *Erwinia herbicola*. The assay provides a rapid and convenient method to screen large numbers of bacteria.

Colorimetric methods have long been employed for the detection of indoleacetic acid (IAA) produced by plants and microorganisms (6). Determination of the IAA-producing capability of a microorganism is useful in its identification and provides a valuable marker when examining the physiological roles or ecological significance of IAA in establishment and persistence of the organism (1, 15). Current methods for the analysis of IAA production by bacteria requires cell-free supernatants or purified extracts (7, 8). These methods are time consuming and complicated, and some require the use of sophisticated instruments. They also require individual testing of each isolate, resulting in a substantial investment of time, labor, and supplies when large numbers of bacteria are analyzed. Although these methods can be very precise, a purely qualitative determination of IAA production may be all that is required to achieve research objectives.

In this paper we report the development of a rapid, specific, and convenient method for the qualitative analysis of IAA production by *Pseudomonas syringae* subsp. *savastanoi* and other bacteria. Large numbers of bacterial colonies can be screened simultaneously with high resolution and accuracy.

(Portions of this work have been reported elsewhere [2].)

MATERIALS AND METHODS

Bacterial strains. Strains of bacteria examined, phenotypes, and origins are summarized in Table 1. Strain PB213 of *P. syringae* subsp. *savastanoi* (Smith) Stevens (*P. savastanoi*), which produces IAA in culture, was used throughout this study. The presence of plasmids encoding IAA production was confirmed routinely by using the alkaline lysis miniprep technique followed by electrophoresis on a 0.5% agarose gel (3, 5, 12). Other bacteria that produce IAA in culture (Iaa$^+$) that were tested in our study include *P. savastanoi* EW2009 and a strain of *Erwinia herbicola* (Lohnis) Dye isolated from oleander. Negative controls included the Iaa$^-$ strains *P. savastanoi* PB213-3 and EW2009-3, an isolate of *P. syringae* pv. syringae van Hall, *Pseudomonas fluorescens* Migula, *Agrobacterium tumefaciens* (Smith & Townsend) Conn., an Iaa$^-$ strain of *E. herbicola*, and *Xanthomonas campestris* pv. juglandis (Pierce) Dowson.

Growth media. Cultures were maintained on amended Luria-Bertani agar medium (LB) containing the following (in grams per liter): Bacto-Tryptone (Difco), 10; yeast extract, 5; NaCl, 5; and Bacto-agar (Difco), 20. The pH was adjusted to 7.5 with 1 N NaOH before autoclaving. LB, LB amended with 5 mM L-tryptophan (LBT), and LBT amended with 0.06% sodium dodecyl sulfate plus 1% glycerol (LBTD4; 9) were used in experiments.

Assay conditions. Agar plates (9-cm diameter) were inoculated with toothpicks into a grid pattern from agar cultures or spread uniformly with a suspension obtained from an extract of inoculated plant leaves. Grid plates consisted of replicated rows of several isolates per plate or a random mixture of strains PB213 and PB213-3. In some experiments replicate plates were prepared for comparison of various media, membranes, reagents, or incubation periods.

Each inoculated plate was overlaid with an 82-mm-diameter disk of one of the following membranes: nitrocellulose (MFS or Amersham), nylon (Amersham), charge-modified nylon (Nytran; Amersham), DEAE cellulose (Schleicher & Schuell) or Whatman no. 1, 2, 5, or 540 paper. Grid plates were overlaid immediately after inoculation. Spread plates were overlaid after an initial incubation period of 24 h to enumerate total bacteria. All plates were incubated until colonies reached 0.5 to 2 mm in diameter. For routine assays, up to 50 CFU per plate could be accurately evaluated. More densely colonized plates were examined by transfer of isolates to fresh plates in a grid format.

After an appropriate incubation period, the membrane or paper was removed from the plate and treated with Salkowski reagent (8). Two formulations of the reagent were tested. Reagent A was 2% 0.5 M $FeCl_3$ in 35% perchloric acid, and reagent B was 1.2% $FeCl_3$ in 37% sulfuric acid. Membranes were saturated in a petri dish by soaking directly in reagent or by overlaying on a reagent-saturated filter paper. Whatman no. 1, 2, 5, and 3MM filter papers were

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TABLE 1. Bacterial species and strains used in this study

Species and strain	Phenotype	Source or reference
P. syringae subsp. *savastanoi* PB213	Wild type, Iaa$^+$	4
P. syringae subsp. *savastanoi* PB213-3	Iaa$^-$	Spontaneous mutant of 213 (4)
P. syringae subsp. *savastanoi* EW2009	Wild type, Iaa$^+$	Oleander strain (14)
P. syringae subsp. *savastanoi* EW2009-3	Iaa$^-$	Spontaneous mutant of EW2009 (14)
P. syringae pv. syringae B-15$^+$	Iaa$^-$	Field isolate from almond (6)
E. herbicola CLPB159	Iaa$^+$	Field isolate from oleander; A. Bishop
E. herbicola B-53	Iaa$^-$	J. DeVay
A. tumefaciens, biotype I, B-208[a]	Iaa$^-$	Tomato; J. DeVay
X. campestris pv. juglandis B-31	Iaa$^-$	Walnut; R. Bostock

[a] This strain does not produce IAA on growth media used in our experiments.

examined. The reaction was allowed to proceed until adequate color developed. All reagent incubations were conducted at room temperature. Bacteria producing IAA were identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony.

Sensitivity and specificity of the assay. A dilution series with concentrations of IAA from 0.05 to 8 nmol per 0.5-μl aliquot was applied directly onto nitrocellulose disks in a grid pattern of two replications of each dilution. A similar grid format was employed in tests that compared specificity of the assay for IAA relative to other indole compounds. Stock solutions of IAA and indole compounds were freshly prepared as ethanol solutions, and dilutions were made in distilled water (IAA) or ethanol (indoles). The resulting spots formed on the nitrocellulose membranes were approximately 2 mm in diameter, approximately the size of bacterial colonies when assayed.

The specificity of the assay was assessed by comparing IAA with indolebutyric, indolepyruvic, and indolepropionic acids as well as the IAA conjugates IAA-alanine, IAA-aspartate, and IAA-glycine. The IAA precursor L-tryptophan was included as a negative control. Each row of the grid format contained four replicates of one compound at a concentration of 1 nmol per 0.25-μl aliquot. All test compounds were purchased from Sigma Chemical Co.

Membranes were assayed on Whatman no. 2 filter pads saturated with Salkowski reagent A. In early trials, membranes were incubated for up to 3 h to allow the possible development of any weakly reactive compounds or dilutions. In later experiments, all membranes were incubated for a minimum of 30 min, which was determined to be the optimum development period for these tests.

Colony hybridization analysis. After the nitrocellulose membranes were removed for IAA analysis, nylon membranes (Nytran) were overlaid on plates with colonies selected for identification by gene probe analysis. These plates were incubated until colonies reached 1 to 2 mm in diameter on the membranes. The plates were then subjected to colony hybridization analysis (12). Briefly, membranes were prehybridized in a solution containing 1.5× SSPE (12), 1% sodium dodecyl sulfate, 0.2% polyanetholesulfonic acid, 50% formamide, and single-stranded salmon sperm DNA for 2 h at 49°C and then incubated overnight in hybridization solution that contained the same components as the prehybridization solution plus 10% polyethylene glycol and 25 ng of DNA derived from the IAA operon of strain PB213 labeled with [^{32}P]dCTP (Amersham) with a BRL random prime kit as recommended by the manufacturer. The probe was a gel-purified 2.8-kb internal *Eco*RI fragment from pLUC2, which includes all of *iaaM* and a portion of *iaaH*, the genetic determinants in *P. savastanoi* for tryptophan 2-monooxygenase and indoleacetamide hydrolase, respectively (4, 10). After removal of excess probe, the membranes were analyzed by autoradiography with Kodak XAR-5 film for visualization.

RESULTS AND DISCUSSION

Assay development and optimization. Colonies of *P. savastanoi* PB213 (Iaa$^+$) immobilized on a nitrocellulose membrane and then treated with Salkowski reagent A produced a deep red ring in the membrane surrounding the colonies (Fig. 1). The color of the reaction is similar to that produced with IAA in solution assays with this reagent (8). The reagent also exhibited a high level of specificity for other IAA-producing bacteria such as *E. herbicola*, and the color reactions (data not shown) with these bacteria were identical to that for PB213. *P. savastanoi* PB213-3 (Iaa$^-$) (Fig. 1) and other bacteria not producing IAA effected no color change in the membrane and were easily distinguishable by visual inspection. No color reaction occurred with a gall-forming isolate of *A. tumefaciens* containing the *tms-1* and *tms-2* genes, which are thought to be required for IAA synthesis in plant tumors and have extensive homology with the *iaaM* and *iaaH* genes of *P. savastanoi* (16). However, this isolate does not produce IAA under the culture conditions used in our experiments.

Direct treatment of an inoculated plate was unsuitable because of acid hydrolysis of the agar gel by the reagent. Transferring colonies to a membrane immobilized the bac-

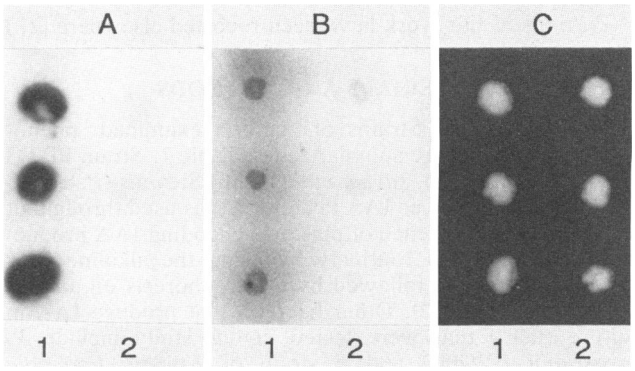

FIG. 1. Visualization of colonies of *P. savastanoi* PB213 (Iaa$^+$; lanes 1) or PB213-3 (Iaa$^-$; lanes 2) on nitrocellulose membranes by autoradiography after hybridization of a membrane with the ^{32}P-labeled DNA probe derived from pLUC2 (A) or after reaction of a membrane with the Salkowski reagent (B). (C) Original colonies on LBTD4 medium from which the lifts were made.

teria on a solid support, maintained their orientation, and preserved the original plate for later colony selection. Optimum results were obtained with nitrocellulose, which exhibited clearly defined reaction sites with little background discoloration (Fig. 1). Whatman no. 1, 2, 5, 540, and 3MM paper disks allowed adequate bacterial growth but proved too porous to limit IAA accumulation to a discrete zone surrounding a bacterial colony. Nylon membranes were cleared by the test reagent, which revealed and discolored the fiber matrix, making interpretation difficult. Charge-modified DEAE-cellulose and Nytran membranes were investigated to determine whether anionic IAA molecules could be bound at the colony site to limit diffusion. This was not the case, perhaps because of the strongly acidic nature of the Salkowski reagent.

We examined various Whatman papers upon which to overlay the membranes and found no. 2 to perform best. Diffusion was limited to the immediate vicinity of the colony, which resulted in a high degree of resolution. Sufficient reagent was absorbed to keep the colony lift saturated throughout color development. Excessive diffusion was avoided by saturating the paper to slightly less than capacity (2.5 ml of reagent for a 9-cm disk). Both formulations of the Salkowski reagent were acceptable, but the perchloric acid reagent proved to be superior in a number of ways. The perchloric acid reagent yields a deep red color at sites of IAA accumulation on the membrane that develops after approximately 30 min and increases in intensity for 4 h, with excellent resolution of Iaa^+ and Iaa^- phenotypes within 1 h. Nonspecific background reactivity is limited to a slight yellowing of the membrane that is apparent several hours after evaluation of IAA. The reaction with the sulfuric acid reagent occurs more slowly and yields a purple hue that is visible after approximately 2 h and gradually darkens. Formation of a pale violet background of the membrane interferes with detection of isolates that produce low amounts of IAA. Additionally, discoloration of bacterial colonies may occur, further complicating analysis. The color reaction with the sulfuric acid reagent requires approximately four times as long to equal the intensity of that with the perchloric acid reagent and never achieves a similar final intensity.

Uniform exposure of colonies with the Salkowski reagent is required for optimum sensitivity. The reagent is somewhat volatile; hence it is important to provide sufficient volume during color development. Insufficient reagent volume resulted in little or no color development with Iaa^+ colonies. Direct saturation of the membrane by floating it on assay solution increased diffusion of IAA from the membrane into the reagent and decreased resolution of colonies. Incubating the membrane above a filter paper saturated with reagent maintained the membrane in a saturated state by wicking the reagent from beneath without allowing excessive diffusion of IAA from the site of production.

Best results were obtained with bacterial colonies of approximately 0.5 to 2 mm in diameter before analysis. This size provided adequate growth for synthesis and excretion of detectable levels of IAA without allowing excessive diffusion of IAA to adjacent colonies.

The presence in the media of the IAA precursor L-tryptophan was essential for this method of detecting IAA production by the bacterial isolates examined. Bacteria produced detectable levels of IAA when grown on media prepared from compounds containing L-tryptophan (e.g., Casamino Acids); however, incorporation of an additional 5 mM L-tryptophan reduced by approximately fourfold the time required to detect IAA and increased the color intensity. The presence of L-tryptophan neither inhibited nor enhanced the growth rate of any bacteria on the media examined. An insignificant yellow background discoloration may form on membranes lifted from media containing added L-tryptophan. This background differs markedly in color and intensity from the pigment produced by IAA and does not manifest itself until well after the development of a reaction with IAA.

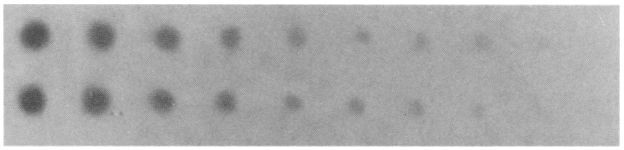

FIG. 2. Sensitivity of the assay to various concentrations of pure IAA. Concentrations of IAA in duplicate spots are (from the left) 8, 4, 2, 1, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 nmol. Although the spots corresponding to 0.05 and 0.1 nmol are barely visible in the photograph, these concentrations are clearly apparent when viewed in color. The high concentrations of IAA produce a deep red color and the lower concentrations yield a pink color after exposure to the Salkowski reagent.

Assay sensitivity and specificity. The minimum detectable level of IAA was approximately 50 pmol in a 2-mm^2 spot (Fig. 2). In these experiments, color development was first visible at the highest IAA concentration within minutes and continued to increase in intensity for a period of 30 min. Concentrations not visible at 30 min did not develop upon further incubation. After 30 min, the color began to fade, so that by 1 h low concentrations (50 pmol) were no longer visible. The highest concentrations of IAA yielded a dark red color, whereas the color gradually faded to pink with dilution.

The reaction was highly specific to IAA, although the IAA analogs IAA-alanine, IAA-aspartate, and IAA-glycine formed dark red, purplish pigments that appeared slightly darker than the color generated by IAA. Color appeared almost immediately from 1-nmol spots of indolebutyric, indolepyruvic, and indolepropionic acids. All three compounds developed yellow to yellow-brown pigments that were clearly different from IAA. Spots of L-tryptophan failed to exhibit any color reaction to the reagent.

Use of the assay for epiphytic population studies. The membrane assay proved to be highly reliable for identifying strain PB213 or EW2009 in greenhouse tests of endophytic populations in oleander and of epiphytic populations of strain EW2009 on strawberries in field tests (unpublished data). Mixed populations of Iaa^+ and Iaa^- bacteria recovered on the same membrane were easily differentiated with the assay (Fig. 1). Colony hybridization analyses confirmed that all colonies producing IAA also hybridized to and gave a strong signal with the DNA probe containing portions of the IAA operon of *P. savastanoi* (Fig. 1). To further verify the fidelity of the assay for detecting IAA production by colonies recovered from a known epiphytic bacterial population, strawberry plants in the greenhouse were inoculated with a spontaneous rifampin-resistant isolate of strain EW2009. The leaves were sampled, and the washings were plated on LBTD4 containing 50 μg of rifampin per ml. All colonies recovered on the plates effected a positive color reaction for IAA in the membrane assay.

A flow chart outlining the principal steps of the IAA assay is presented in Fig. 3. We have found this assay to be essential for simultaneously screening large numbers of

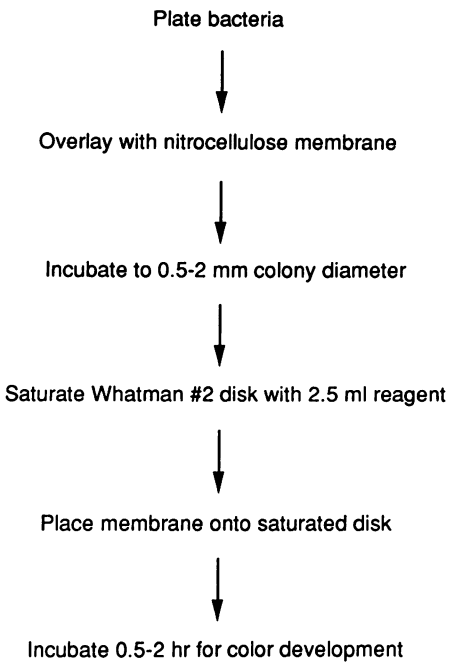

FIG. 3. Flow chart of in situ analysis for IAA production.

bacteria isolated from plants in studies of the establishment and persistance of epiphytic and endophytic *P. savastanoi* (13). In addition, we have used this method for the detection of IAA-producing pseudomonads recovered from blossoms of French prune (unpublished data). Another simple method for the detection of certain pseudomonads on plant tissues relies on their ice-nucleating ability (11). The assay reported in this paper provides a useful addition to the methods available for the detection and enumeration of *Pseudomonas* spp. and other bacteria for which IAA production is an appropriate criterion.

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REFERENCES

1. **Bric, J., R. Bostock, and J. Marois.** 1988. Abstr. Intl. Conf. on Risk Assessment in Agricultural Biotechnology.
2. **Bric, J. M., and R. M. Bostock.** 1988. Phytopathology **78**:1609.
3. **Comai, L., and T. Kosuge.** 1980. Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in *Pseudomonas savastanoi*. J. Bacteriol. **143**:950–957.
4. **Comai, L., and T. Kosuge.** 1982. Cloning and characterization of *iaaM*, a virulence determinant of *Pseudomonas savastanoi*. J. Bacteriol. **149**:40–46.
5. **Comai, L., G. Surico, and T. Kosuge.** 1982. Relation of plasmid DNA to indoleacetic acid production in different strains of *Pseudomonas syringae* pv. *savastanoi*. J. Gen. Microbiol. **128**: 2157–2163.
6. **DeVay, J. E., F. L. Lukezic, S. L. Sinden, H. English, and D. L. Coplin.** 1968. A biocide produced by pathogenic isolates of *Pseudomonas syringae* and its possible role in bacterial canker disease of peach trees. Phytopathology **58**:95–101.
7. **Fett, W., S. F. Osman, and M. F. Dunn.** 1987. Auxin production by plant-pathogenic pseudomonads and xanthomonads. Appl. Environ. Microbiol. **53**:1839–1845.
8. **Gordon, S. A., and R. P. Weber.** 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. **26**:192–195.
9. **Kado, C. I., and M. G. Heskett.** 1970. Selective mediia for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. Phytopathology **60**:969–976.
10. **Kosuge, T., M. G. Heskett, and E. E. Wilson.** 1966. Microbial synthesis and degradation of indole-3-acetic acid. J. Biol. Chem. **241**:3738–3744.
11. **Lindow, S. E., S. S. Hirano, W. R. Barchet, D. C. Arny, and C. D. Upper.** 1982. Relationship between ice nucleation frequency of bacteria and frost injury. Plant Physiol. **70**:1090–1093.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Silverstone, S. E., R. M. Bostock, D. G. Gilchrist, and T. Kosuge.** 1990. Phytopathology **80**:972.
14. **Smidt, M., and T. Kosuge.** 1978. The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of *Pseudomonas savastanoi* in gall formation on oleanders. Physiol. Plant Pathol. **13**:203–214.
15. **Surico, G., L. Comai, and T. Kosuge.** 1984. Pathogenicity of strains of *Pseudomonas syringae* pv. *savastanoi* and their indoleacetic acid-deficient mutants on olive and oleander. Phytopathology **74**:490–493.
16. **Yamada, T., C. Palm, B. Brooks, and T. Kosuge.** 1985. Nucleotide sequences of the *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. Proc. Natl. Acad. Sci. USA **82**:6522–6526.